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SPERM PRECEDENCE IN EXPERIMENTAL INTERSPECIFIC MULTIPLE MATINGS OF HYBRIDIZING NORTH AMERICAN TIGER SWALLOWTAIL BUTTERFLY SPECIES (LEPIDOPTERA: PAPILIONIDAE)

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ABSTRACT. *Papilio canadensis* and *P. glaucus* have multiply-mating females and males. Laboratory hand-pairings allowed us to by-pass natural mating preferences of polyandrous females and conduct sequential (twice-mated) lab pairings; one heterospecific and one conspecific male for each virgin female of *P. glaucus* or *P. canadensis*. Using electrophoresis and species-specific (diagnostic) allozymes, we were able to determine whether random fertilization (a mix of heterospecific sperm) or sperm precedence (either first or last male) existed for these twice-mated females. Since the number of useful double pairings for lab and field female analyses was low, we can only conclude that of double mated females, most are fertilized by males of their first mating, but that a mix of sperm and last male sperm precedence do occur at lower frequencies.

Overall, these lab results suggest that if it exists, sperm selection by females of *P. glaucus* and *P. canadensis* would not necessarily provide a clear mechanism of reproductive isolation. In fact, eggs from interspecific primary hybrid lab crosses (73 families of *Pg* × *Pc* and 17 families of *Pc* × *Pg* hybrids; all single pairing F_1 crosses) are as fertile and viable as those from parental types. The isolating mechanisms that maintained a narrow hybrid zone from 1980-1997 have not been sustained during the recent 1998-2003 period where extensive interspecific hybrid introgression and X-chromosome recombination has occurred. It is clear that even though lab-reared males were regularly fed a solution of honey water, amino acids, and electrolytes, they were less successful at producing larval offspring that were field-captured (wild) males when mated to virgin females. This difference in reproductive success mandates caution in extrapolation from lab to field studies. However lab-reared males can show first male or last male sperm precedence. It is also possible for a female to have a mixture of fertilizations from males of different species. We are therefore hesitant to interpret these preliminary results with relatively small sample sizes in the context of sexual selection theory. These results do complicate the interpretation of hybrid zone dynamics.

Additional key words: Conspecific sperm, *Papilio glaucus, P. canadensis*, polyandry, hybrid zone spermatophore..

INTRODUCTION

Females of many animal species mate multiple times within their fertile lifetime (Smith 1984; Birkhead and Møller 1998). There is evidence that these multiple matings may extend female life-span (Wagner *et al.* 2001), increase egg production (Wiklund *et al.* 1993; Boggs 1997a, 1997b, Karlsson 1998, Eady *et al.* 2000), and increase offspring viability (Lederhouse and Scriber 1987; Tregenza and Wedell 2002). It may also increase offspring weight/size (Sakaluk *et al.* 2002) or offspring reproductive success (Bernasconi and Keller 2001; Pai and Yan 2002). However, where males are of different species, egg production can be drastically reduced (Shapiro 2000) or post-zygotic hybrid offspring survival may be poor (Orr and Turelli 2001; Marshall *et al.* 2002). Mixtures of species diagnostic traits from a single mother could result from fertilization by both heterospecific and conspecific males in a hybrid zone. Clarification of sperm precedence for naturally hybridizing species is needed.

Sperm are stored for use in fertilization, sometimes for the entire life of the insect, in female organs called spermathecae (Cook and Wedell 1996; Gage 1998). Some species with different types of sperm storage organs (Pitnick *et al.* 1999) can separately store sperm from different males, as in the yellow dung fly (Ward 1998; Hellriegel and Bernasconi 2000). This creates the possibility that sperm from two (or more) different males are in competition for egg fertilizations, extending sexual selection beyond copulation. Packets of sperm (spermatophores) are transferred to polyandrous females of the North American tiger swallowtail butterfly species group, raising questions of sperm precedence and sperm competition from different mates.

Females of *Papilio glaucus* L. and *P. canadensis* R & J, often mate four to five times during their relatively short lifetime of three to six weeks (Lederhouse *et al.* 1989; Lederhouse 1995; Scriber *et al.* 1998a) and males can mate at least four times (Lederhouse 1995). We have shown that fertility declines in *P. glaucus* females after seven to eight days and that a second mating may restore it (Lederhouse and Scriber 1987; see also Watanabe 1988). This may be the result of sperm depletion or other factors.

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As in many Lepidoptera, these two species of *Papilio* usually package sperm in a spermatophore, which is energetically costly (Mann 1984; Watanabe and Hirota 1999). Sperm are often dependent upon the female's muscles to move them out of the spermatophore and transfer them to the spermatheca (Tschudi-Rein and Benz 1990). While the pattern of paternity in butterflies and moths is variable, it is believed that sperm from one male takes precedence. However, it might be an earlier mate instead of the most recent one that sires most or all of the offspring (Clarke and Sheppard 1962; Flint and Kressing 1968; Pair *et al.* 1977; Bissoondath and Wiklund 1997; Cook *et al.* 1997; Wedell and Cook 1998; LaMunyon 1999). The importance of which male's sperm sires offspring may be of even greater ecological significance when males are from different species, as can occur in hybrid zones of parapatric species.

Multiple-mating females and males of tiger swallowtail butterflies may encounter heterospecific mates as well as their conspecifics in the vicinity of hybrid zones such as the one across the Great Lakes region (generally at latitudes of 40-43°N) from Minnesota through Wisconsin, Michigan, New York State, and New England (Scriber 1996; Scriber *et al.* 2003). We have documented such parapatric range overlap and cases of natural hybridization of the species in the field (Luebke *et al.* 1988; Scriber 1990; Deering and Scriber 1998; Scriber *et al.* 1998b; Stump 2000; Ording 2001; Donovan and Scriber 2003; Hereau and Scriber 2003). Mating preferences of both *P. glaucus* and *P. canadensis* males in the field are both very strong for tethered *P. glaucus* females in 2-choice tests using size-matched, tethered virgin females, which represents a strong and unexpected asymmetry (Deering 1998; Deering and Scriber 2002). Females were capable of free flight on the thread tethers and were able to reject some male mating attempts. However, few rejections were observed for these lab-reared virgin females. No evidence of sex pheromones has been detected in field tests with cryptically caged virgin females compared to empty control cages behind dead display females (Deering 1998).

Interspecific hybridization for *P. glaucus* and *P. canadensis* has been documented to increase extensively within and north of the historical hybrid zone during the recent warming climatic trends during 1998–2003 (Scriber 2002a; Scriber and Ording 2005). The potential of post-mating (but prezygotic) reproductive isolation had not been examined for these butterflies (e.g. differential selection of sperm by females). In particular, we wondered whether multiple-mating females of *P. glaucus* or *P. canadensis* may cryptically select sperm of conspecifics over heterospecifics as suspected in grasshoppers (Hewitt *et al.* 1989; Bella *et al.* 1992), crickets (Gregory and Howard 1994; Howard *et al.* 1998), *Drosophila* (Price 1998), ladybird beetles (Katakura 1986b), and flour beetles (Wade *et al.* 1993). If so, strong conspecific sperm precedence could help explain why the hybrid zone has remained basically geographically distinct and ecologically stable for the past two centuries (Scriber *et al.* 1982, 1996) despite the capacity for strong flight and long range dispersal of both species (Scriber *et al.* 1998b).

We employed species-specific diagnostic allozymes to identify the paternity of larvae produced after two sequential hand pairings of individual virgin females with heterospecific and conspecific (or vice versa) males of *P. glaucus* and *P. canadensis*. We also compared males of the more distantly related *P. eurymedon*, *P. rutulus*, *P. multicaudatus*, and *P. troilus* (Hagen and Scriber 1991). The western species (*P. eurymedon*, *P. rutulus*, *P. multicaudatus*) also hybridize to various degrees (Sperling 1990, 2003; Scriber *et al.* 1995; Layberry *et al.* 1998; Guppy and Shepard 2001). Different benefits of multiple matings (accessory gland substances promoting increased egg production, ejaculates with nutritional benefits; Arnqvist and Nilsson 2000) may result from repeated mating with the same male versus different males (where indirect genetic benefits may also occur; Jennions and Petrie 2000; Zeh and Zeh 2001). The importance of comparing laboratory versus natural polyandrous matings (where females choose their mates and thus influence both the direct and indirect benefits; see Tregenza *et al.* 2003) has recently been emphasized for crickets (Sakaluk *et al.* 2002). Therefore, in addition to lab pairings with virgin females, wild allopatric females of *canadensis* and *glaucus* were captured and subsequently remated to heterospecific males after first collecting her field-fertilized (presumably conspecific) eggs for 1-3 days. Directional postcopulatory sexual selection (involving both sperm competition, where sperm from different males compete to fertilize eggs, and cryptic female choice, where females bias sperm use toward particular males) has been revealed by using artificial insemination in guppies to bypass the complications with natural mating preferences and mating order of polyandrous females (Evans *et al.* 2003). We have functionally accomplished the same thing with laboratory hand-pairing techniques for *Papilio*.

All female butterflies of the *P. glaucus* group are presumably polyandrous and store sperm in spermathecae . We hoped to determine whether sperm from either first or last males have precedence, whether the female may exert cryptic sexual selection of

conspecific sperm for fertilizing her eggs, or if a mixture of sperm from both species (with potential competition) was involved. In addition to being the first such study in these species, the implications of the results for hybrid zone dynamics and the interpretation of the genetics of offspring from wild-caught females could also be significant. From a single mother of hybrid zone origins, a mixture of odd segregating traits in offspring might be explained by multiple matings with males of different species (Clarke and Sheppard 1962).

Methods

Pairings. All double pairings were conducted during the summers of 1996, 1997, and 1998 (Tables 1–4). Both wild-caught and lab-reared male and female butterflies were used for pairings. Females and males of *P. glaucus* (G) and *P. canadensis* (C) and males of *P. rutulus* (R), *P. eurymedon* (E), *P. multicaudatus* (M) were used. Lab-reared, newly eclosed, virgin females were hand-paired to males and allowed to oviposit in multichoice plastic arenas lined with host plant foliage as previously described (Scriber 1993). These were remated (after two to six days and having laid at least 20 eggs), again by hand pairing, to a male of a species different from that of the first male. Females were then allowed to oviposit again. Additionally, wild caught females were allowed to oviposit in plastic boxes, then remated after one to five days by hand-pairing to a male of a different species, and allowed to oviposit again. Only females that were actively laying eggs were remated. The duration of pairings was recorded, as transfer of spermatophores takes at least 30 minutes.

For the *P. glaucus* species group, larvae from eggs laid both before and after rematings were collected and reared on Wild Black Cherry (*Prunus serotina* Ehrh.), a common host of both *P. glaucus* and *P. canadensis*. Larvae of *P. troilus* were reared on *Sassafras albidum* Nutt. or *Lindera benzoin* (L.) Blume (Lauraceae). After reaching approximately the third instar, larvae were frozen at -80°C. Adults were frozen before or immediately after death. Females were later dissected to determine how many spermatophores were present at death. We include females that after dissection only had a single spermatophore (Tables 3 & 4), because we wanted to determine which of the two males passed it. However, we deleted these pairings in our summary of "last" (P2 = 1) or "first" (P2 = 0) sperm precedence. We are also aware that sperm transfer without leaving recognizable spermatophores can rarely occur in Lepidoptera (Cordero 1999). Analyses of egg fertility and viability of a set of single lab-paired females from 1996-1999 with no spermatophores (n=45) were compared to those with one present (n=274).

Female butterflies were fed a 20% honey solution and males were fed a 20% honey solution supplemented with amino acids and salts following Lederhouse *et al.* (1990). Lab-reared males were not paired until at least two days following adult eclosion. For comparison, various other single male pairings among these species were conducted (1996–1999). Comparisons of egg and larval offspring production from field-captured females for various species were also included from years 1983–2002. A separate group of primary (F_1) hybrid crosses between *P. canadensis* and *P. glaucus* from 1995–2002 were compared to the reproductive fitness of wild females of both species (Table 8) to provide a comparison of fecundity and viability.

Allozyme Electrophoresis. Electrophoresis was carried out on thin-layer cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, TX), using methods of Hagen and Scriber (1991). Small larvae were homogenized whole in buffer while the head and thoraxes of larger larvae were homogenized in buffer. The distal half of the abdomen of adult males and the proximal half of the abdomen of adult females was used since the distal part of the female abdomen included male spermatophores. The enzyme 6-phosphate dehydrogenase (*Pgd*) was used to determine differences between the species of the *P. glaucus* species group since all 5 species are fixed for different diagnostic alleles (Hagen and Scriber 1991). Other enzymes with diagnostic differences between species were not used because those alleles were too faintly stained in larvae. Staining of *Pgd* was fainter for larvae than adults, but it was clear and interpretable. In other Lepidoptera, fixed allozyme differences have also been used to identify egg, larval, pupal, and adult species (Woods *et al.* 2001). Paternity of offspring produced after remating of our *Papilio* was established by determining alleles of *Pgd* allozymes of larvae produced after remating. In several cases, the males were lost (or accidentally not frozen soon enough) and could not be evaluated. Precedence for each female was expressed as P2, the proportion of larvae produced after the remating that were sired by the female's second mate.

Results

Lab and wild pairings. Out of 82 interspecific double pairings for lab-reared females, only 32 produced larvae both before and after being remated (Table 1). The others either laid no eggs after being remated, laid no hatching eggs (all being either infertile or fertile and non-viable) after remating, or had laid no hatching eggs before being remated. Of the 32 females producing larvae both before and after being remated, five had been mated to males that shared allozymes.

Some interspecific introgression (or ancestral low-level polymorphisms, or residuals from earlier north/south Pleistocene hybrid zone movement) is found at the *Pgd* locus (Hagen and Scriber 1991; Scriber 1996; Scriber *et al.* 1998b) making the determination of paternity for offspring impossible in these cases, leaving 27 broods where P2 was determined. Of these females, 6 had only a single spermatophore recovered, and 3 others produced fewer than 5 larvae, leaving only 18 for P2 analyses. Since the sample sizes were so small, we did not conduct t-tests.

In addition to the sequential pairings of various lab-reared virgin females (Table 1), wild females were also set up in oviposition arenas and later remated to heterospecific males (Table 2). Out of 27 wild females, 20 produced larvae both before and after being remated. All of the remated wild females produced larvae before being mated. Only one female that had produced larvae after being remated had a P2 that was not possible to be determined electrophoretically, leaving 19 broods where P2 was determined (Table 2). Of these 19 females, 5 had only one (or no) spermatophores and another 7 had fewer than 5 larvae, leaving 7 for P2 allozyme analyses. A shared host plant (*Prunus serotina*) was used to minimize any differential mortality of larvae before the third instar samples for allozyme analysis, but this delay from egg hatch to the third instar, when allozyme samples were made, nonetheless may have introduced an unknown degree of selection bias due to differential mortality among genotypes. Larvae had allozymes corresponding to their parents in all of the cases where allozymes of parents (a once-mated female and her mate) were compared to larval offspring allozymes. We used *Pgd*, which is X-linked in *P. glaucus* and *P. canadensis*. Male homozygotes of *Pgd* could not be distinguished from female hemizygotes in larvae since the sex was not ascertained. The sample numbers are not high enough to be able to estimate accurately the actual population allele frequencies, but there is no reason to suspect non-Mendelian inheritance.

Of the 18 double-paired interspecific lab crosses examined, 11 showed first male sperm precedence (P2 = 0), 4 showed mixed parentage (P2 = 0.20 - 0.93), and 3 showed last male sperm precedence (P2 = 1). Only one of these three last male fertilizations was heterospecific in paternity (Table 3). Of the wild females remated to different species in the lab, 3 showed mixed parentage (from both males; P2 = 0.07-0.14) and the other 4 all showed first male sperm precedence (P2 = 0; Table 4). The number of useful double male pairings was insufficient to evaluate whether female selection of conspecific sperm was possible or not.

Durations of second pairings were recorded. No second pairings lasting less than 30 minutes resulted in sperm replacement. This is not surprising based on time needed for ejaculate transfer in other Lepidoptera (Watanabe and Sato 1993). However, most second pairings lasted for longer than 30 minutes and most females were found to be carrying two spermatophores (or more for wild caught females).

Singly-mated females illustrate the need for a minimum of 30 minutes of copulation duration for successful fertilization (Table 5). A surprisingly high number of females laid eggs even when no spermatophore was present (0.62, n = 45) compared to those with a spermatophore (0.75, n=274, Chi-square 3.32, n.s.). However, only a very small portion of matings with no spermatophores detected produced larvae (0.08, n=24) compared to those with a spermatophore (0.57, n=189, Chi-square 19.87, $p < 0.001$).

Even with regular feedings of males with a honey water/electrolyte "elixir" (as in Lederhouse *et al.* 1990), it is evident that lab-reared males are less successful at producing broods with larvae than are wild field-collected males (Table 6). An index of reproductive success also shows that primary F_1 interspecific hybrids between *P. glaucus* and *P. canadensis* parents (*Pc* × *Pg* and *Pg* × *Pc*) have offspring success that is intermediate compared to the parental types (Table 6).

Average hatchabilities of eggs (# larvae/total. eggs) from field-captured females of these 5 species (ranging from 0.43 to 0.70; Table 7) were comparable to our lab-reared virgin females in these experiments (0.25 to 0.72; Table 6). The proportions of females that oviposited ranged from 33% to 84% among the 7 *Papilio* species. The percentage producing larvae ranged from only 16% to 52% (Table 7). In a separate study of singly-mated females that did produce larvae (from 1995-2002), it was clear that the heterospecific hybrids between *P. glaucus* and *P. canadensis* were at no obvious selective disadvantage compared to the parental types; with mean egg viability of 68.0% (*Pc* × *Pg*) and 62.3% (*Pg* × *Pc*) compared with 58.9% for *P. canadensis* and 65.3% for *P. glaucus* (Table 8).

DISCUSSION

Sperm precedence. Second male precedence has been considered to be the rule in Lepidoptera (Drummond 1984), but our results showed 3 of 25 successful double interspecific matings complete second male sperm precedence (P2 = 1) in these tiger swallowtail butterflies (Tables 3 & 4). Two additional twice-mated females showed a large proportion of

TABLE 1. Types and number of double-paired *Papilio* females and reproductive success following remating. P2 is calculated as the proportion of larvae produced after a second mating that were sired by the female's second mate.

Double-pairing type (female × male 1 × male 2)	double-paired females	laying eggs after remating	producing larvae after remating	producing larvae before and after remating	producing larvae before and after remating where P2 could be determined
(C × C × G)	18	17	8	6	5
(C × G × C)	13	12	6	5	5
(G × G × C)	17	13	9	6	6
(G × C × G)	20	18	13	8	5
(C × C × E)	7	5	2	2	2
(C × E × C)	4	4	2	2	2
(G × G × M)	1	1	1	1	1
(G × M × G)	1	1	1	1	0
(G × R × G)	1	1	1	1	1
Total	82	72	43	32	27
		72/82 = 87.8	43/72 = 59.7	32/43 = 44.4	27/32= 84.4 %

C = *canadensis*, G = *glaucus*, M = *multicaudatus*, R = *rutulus*, E = *eurymedon*

TABLE 2. Types and number of remated wild-caught *Papilio* females and reproductive success following remating.

Double-pairing type (female wild × male)	Number remated wild-caught	Laying eggs after remating	Producing larvae after remating	Producing larvae before and after remating	Producing larvae before and after remating where P_2 could be determined
P. canadensis wild x *P. glaucus* (C wild × G)	8	7	6	6	6
(G wild × C)	2	2	2	2	2
(G wild ×E)	7	6	6	6	5
(G wild × M)	4	3	3	3	3
(G wild × R)	6	3	3	3	3
Total	27	21	20	20	19
		21/27 = 77.8	20/21 = 95.2	20/20 = 100	19/20 = 95%

E, M, R = *eurymedon*, *multicaudatus*, & *rutulus*, respectively

mixed sire offspring (P2 = 0.82 and 0.93) and 5 others showed less extensive mixing (P2 = 0.07-0.36). This pattern appears to be in contrast to the last mate sperm precedence believed to be typical of insects in general (Smith 1984) and especially Lepidoptera. All but one of the 20 Lepidoptera species studied have mean P2 values greater than 0.47 and most are greater than 0.60 (see Table 2.3 in Simmons 2001); although it is reported that distributions are bimodal with 0 or 1 values for most species. Lepidoptera having bimodal distributions create intermediate mean values of P2, and that is one reason why P2 analyses are not very useful in understanding mechanisms of sperm competition (Simmons and Siva-Jothy 2001). Last male sperm precedence occurred (at least partially) in only 10 of 25 double matings for which we could analyze diagnostic allozymes. However, offspring sired by the last male mate occurred in both *P. canadensis* and *P. glaucus* females even when this male was heterospecific (*canadensis*, *glaucus*, and *eurymedon*; Tables 3 & 4). In addition to these inconclusive results, the last male sperm precedence patterns in other insects is also known to break down when a third male is involved (Zeh and Zeh 1994) as is the case with many older *P. glaucus* and *P. canadensis* females (Lederhouse and Scriber 1987; Lederhouse *et al.* 1989; Lederhouse

TABLE 3. Last male sperm precedence , P2, the proportion of offspring that were sired by the second male, for double-paired lab-reared females. Also indicated are the number of larvae produced after remating that had paternity determined by electrophoresis (N), origin of the male used for remating, the days between pairings, the duration of the remating, and the number of spermatophores present in the female at death.

Type (female × male × male 2)	Male origin	Days between pairings	Duration of remating (minutes)	Spermatophores present	P2	N
C× C × G	lab	3	65	2	1	15
	lab	3	100	2	0	27
	lab	2	26	1	0	61
	lab	3	60	2	0.2	5
C × G × C	lab	3	59	2	0	7
	lab	3	35	1	0	22
	lab	2	>43	2	0	12
	wild	4	57	2	1	21
G × G × C	lab	5	93	1	0	16
	lab	2	>36	2	0	21
	lab	2	>38	2	0	23
	lab	3	62	2	0.36	11
	lab	3	63	2	0	26
	lab	3	106	1	0	14
G × C × G	lab	3	>30	2	0.82	11
	lab	4	85	2	0	72
	wild	2	87	2	0	26
	wild	4	>30	2	0	23
C × C × E	wild	4	>41	2	0	21
	wild	2	73	2	0.93	14
C × E × C	wild	2	>85	1	0	21
	wild	2	>91	2	1	5
G ×R × G	wild	1	99	2	0	19

1995).

The relatively high success of first matings compared to the second matings in our studies is due to unknown factors. One possibility is simply that we did not wait long enough after the first pairing for natural decline in fertility or sperm volume before the second mating was made. For example, Lederhouse and Scriber (1987) have shown that fertility of these species declines over the first week after mating, but that the decline is most rapid after 6–10 days for *P. glaucus* and after 4–7 days for *P. canadensis*. Females in the field may normally reject second males for a longer period of their life than was the case for our experimentally forced laboratory hand pairings. It is also possible that the second male's sperm may have had insufficient time to reach the spermatheca of females in 2–3 days. Another area that deserves additional experimentation would be the assessment of whether males prevented from their

TABLE 4. Sperm precedence P2 for remated wild-caught females. Also indicated are the number of larvae produced after remating that had paternity determined be electrophoresis (N), origin of the male used for remating, the days between collection of the female and remating, the duration of the remating, and the number of spermatophores present in the female at death.

Type(female wild × male)	Male origin	Days until remating	Duration of remating (minutes)	Spermatophores present	P2	N
C wild × G	wild	3	43	2	0	7
	wild	4	33		0	14
	wild	3	60	3	0.14	7
	wild	3	72	3	0	5
G wild × C	lab	2	42	1	0	20
	lab	2	49	2	0	20
G wild × E	wild	1	>40	4	0.07	14
	wild	1	54	2	0.12	8
	wild	4	64	1	0	22
	wild	4	>75	2	0	21
G wild × R	wild	1	27	1	0	11

natural choice of female mates (in hand-paired cases) would withhold or reduce the amount of sperm transferred in laboratory pairings compared to natural matings. Few studies exist of sperm utilization patterns in nature (Cobs 1977; Allen *et al.* 1994; LaMunyon 1994). However, recent research shows that male ejaculate expenditures are dynamic and that males assess the mating status and relative fecundity of females and subsequently modulate the quantity or quality of ejaculate they pass to females (Wedell *et al.* 2002). This also exists as a possible explanation of our poor second mating success.

The roles of apyrene (lacking a nucleus) and eupyrene (with nucleus) sperm in competition (Cook and Wedell 1996, 1999; Watanabe *et al.* 2000) are completely unknown for these two *Papilio* species, and knowing these might help clarify results such as ours. The degree of polygamy and polyandry will vary with age, extent of protandry, the size of spermatophores, as well as species characteristics, all of which makes understanding sperm precedence a complex venture with slowed progress (Kempanaers *et al.* 2000; Pitnick and Brown 2000; Wiklund 2003).

The females that continued to produce fertile eggs and larvae sired by an earlier male (70% of those shown in Tables 3 and 4) also did so even when this original mate was a heterospecific male (followed in mating sequence by a conspecific male). This further suggests that these *Papilio* females may not be exerting active or passive conspecific sperm selection (Birkhead 2000). It has been suggested that genetic benefits from mate choice may be context-specific (Qvarnström 2001). In any case, it is feasible that sperm competition could override any cryptic female selection should it be found to occur.

The failure of many second matings to sire offspring in these interspecific pairing sequences is likely due to some of the same problems encountered in first pairings (Stump 2000). For example, as with first pairings, it appears that second pairings lasting at least 30 minutes are required for sperm replacement (Table 3). We found in *Papilio* single mating studies (Table 6) that only about 25-45% of hand pairings gave rise to some larvae (24.0% *canadensis* × *canadensis*, n=75; 32.4% *glaucus* × *glaucus*, n=33; 42.1% of *glaucus* x *canadensis* hybrids, n=38; and 44.0% of *canadensis* × *glaucus* hybrids, n=50: Stump 2000). Previous studies showed that in 1986 only 19% of 75 pairings produced some larvae (14 fertile females averaging 27 larvae from 73 eggs) After feeding males an elixir solution of amino acids, electrolytes, and sugar our results improved to 90% of 20 females producing some larvae (18 fertile females averaging 44 larvae from 105 eggs; Lederhouse *et al.* 1990).

We do not know the explanation for seemingly low percentages of mating success, but it is reasonable to assume that similar problems may explain at least a portion of the low second mating "replacement" success (70% of females continued to use earlier sperm). Wild

TABLE 5. Success of single mate pairings as indicated by those with viable larvae, grouped by mating duration. The pairings[1] were of various types (conspecific, heterospecific, backcross, and F_2), involved various *Papilio* species (*P. canadensis, P. glaucus, P. eurymedon, P. rutulus, P. multicaudatus, and P. troilus*), and were between lab-reared virgin females and either lab-reared or wild-caught males. Sample sizes for each duration/category are indicated in parentheses. Not all females were dissected and examined for a spermatophore.

Duration (minutes)	Proportion of pairings with a spermatophore[2]		Proportion of all pairings producing eggs		Proportion of pairings with larvae (of those with eggs)	
<30	0.0	0 / 10	61.1	11 / 18	0.0	0 / 11
30-90	94.6	210 / 222	75.3	223 / 296	59.2	132 / 223
>90	76.7	23 / 30	78.7	37 / 47	56.8	21 / 37

[1]all 1996-1999 laboratory hand-pairings of various *Papilio* populations or species were included.
[2]Some females were not examined for spermatophores

females of the 7 species also had low proportions successfully ovipositing and producing larvae (Table 7). Rather than sperm competition, our experimental procedures (second matings after only 2–6 days) may be a partial explanation for our generally low second male fertilization success. Waiting 7–8 days might better reflect the natural remating behavior and physiological. needs of females. Remating interval and spermatophore size can also affect the P2 values and number of matings for Lepidoptera (Drummond 1984; Lederhouse *et al.* 1989; Simmons and Siva-Jothy 2001).

First or last male sperm precedence may occur and be operational in different ways for multiple matings and be limited to only conspecific males (or only heterospecific males). In other species, viability of offspring has been shown to increase with additional conspecific male mates by avoiding the costs of inbreeding (Tregenza and Wedell 2002) or by gaining other genetic benefits (Yasai 1998; Newcomer *et al.* 1999; Jennions and Petrie 2000; Zeh and Zeh 2001; Sakaluk *et al.* 2002). Our experiments involved only combinations that used male mates from two different species. No double conspecific matings were made in this study. With hand-pairings, females were unable to make natural mate choices seen for females with free choice either in the lab or in the field (which can also vary; Sadek 2001).

Males of various insect species may attempt to guard their mates from competing males (Birkhead and Parker 1997) or to secrete mating plugs to block competitors access to the female's reproductive tract (Thornhill and Alcock 1983; Orr 1995). In addition, males may attempt to gain an advantage over potentially competing sperm by a number of mechanisms. First, there may be removal of previous male sperm (Lefevre and Jonsson 1962; Waage 1979; Ono *et al.* 1989; Orr 1995; Price *et al.* 2001). Secondly, hindering or rendering rival sperm non-functional with seminal secretions may occur (Katakura 1986a; Katakura and Sobu 1986; Harshman and Prout 1994; Clarke *et al.*

TABLE 6. Index of mating success for single mate pairs (1996-1999). For each pairing type, this is calculated as the proportion of pairings producing hatching larvae multiplied by the average hatchability of broods producing larvae. Female listed first in each pairing type.

Pairing Type (Female × Male) origin	(n)	Of pairings lasting at least 30 minutes, proportion producing larvae	Average egg hatchability of pairings producing larvae	Index of mating success
C ×C wild	(17)	0.45	0.49 ± 0.06	0.22
C × C lab	(7)	0.11	0.40 ± 0.12	0.04
G × G wild	(1S)	0.78	0.72 ± 0.13	0.43
G × G lab	(18)	0.42	0.63 ± 0.07	0.26
C × G wild	(22)	0.52	0.61 ± 0.06	0.32
C × G lab	(10)	0.33	0.47 ± 0.10	0.16
G × C wild	(37)	0.59	0.66 ± 0.04	0.39
G × C lab	(4)	0.20	0.28 ± 0.09	0.06
T × G wild	(6)	0.67	0.25 ± 0.03	0.17
(C × G) × C wild	(17)	0.52	0.37 ± 0.06	0.19

(C × G) × C are backcross broods using hybrid females and wild *canadensis* males.
T = *P. troilus*

TABLE 7. Pooled total laboratory lifetime egg and larval production of wild field-collected *Papilio* females of the tiger swallowtail (*glaucus*) species group.

	Female Families			Population traits		
Species	**Total (n)**	**with Eggs**	**with Larvae**	**Total eggs**	**total larvae**	**hatchability (l/e)**
canadensis	730	353 (48%)	230 (32%)	13205	6311	.478
glaucus	959	524 (55%)	369 (38%)	31806	17341	.545
rutulus	65	43 (66%)	33 (51%)	1479	795	.538
eurymedon	33	11 (33%)	5 (16%)	297	172	.579
multicaudatus	25	21 (84%)	13 (52%)	599	259	.431
alexiares	31	19 (61%)	12 (38%)	919	475	.517
troilus	88	55 (63%)	35 (39%)	4417	3083	.698

P. canadensis and *P. glaucus* (and *P. alexiares*) data are from 1983-1986 only (others all include 1991-2002 individuals as well as 1983-1986).

1995; Rice 1996; Price *et al.* 1999, 2000). Thirdly, diluting or delaying competing sperm with sperm of higher volumes may be used (Sugawara 1979; Dickinson 1986; Gage and Baker 1991; Karlsson 1995; Gage and Barnard 1996; Simmons and Siva-Jothy 1998). Fourthly, transferring sperm or spermatophores of higher quality or effectiveness may give an advantage (Bertran *et al.* 1996; Smedley and Eisner 1996; Vahed 1998; LaMunyon and Ward 1999; Gilchrist and Partridge 2000; Stjernholm and Karlsson 2000; Price *et al.* 2001; Bergstrom and Wiklund 2002). Finally, there may be production of high volumes of non-nucleated apyrene sperm, which may prevent fertilization by eupyrene sperm of former males (Silberglied *et al.* 1984; Watanabe *et al* 2000). However, since some females may potentially manipulate sperm storage and use (Birkhead *et al.* 1993; Sakaluk and Eggert 1996; Siva-Jothy and Hooper 1996; Otronen 1997; Wilson *et al.* 1997; Neubaum and Wolfner 1999; Olsson *et al.* 1999) to give certain types of sperm a potential competitive advantage (e.g. to conspecific males over heterospecific males) (Robinson *et al.* 1994; Albuquerque *et al.* 1996; Eberhardt 1996; Howard 1999), the particular mechanisms determining which sperm wins is difficult to discern. It may relate to the nature of seminal fluids (Price 1997; Simmons 2001) or of the deposition patterns in all female storage components (Ward 1993). Also, both male and female processes may interact to produce sperm displacement or to affect the outcome of sperm competition (Clark *et al.* 1999; Simmons *et al* 1999; Eberhard 2000; Ward 2000; Simmons 2001). However, males attempting to increase their own fertilization success can result in a decrease of female fitness, producing sexual conflict (Stockley 1997; Simmons 2001; Chapman *et al.* 2003; Pizzari and Snook 2003). At a local population level, such male-female conflict within a species can lead to the rapid evolution of reproductive isolation (Tregenza 2003; Martin & Hosken 2003). The role such sexual selection may have in the reproductive isolation of hybridizing species such as these *Papilio* is currently unknown but may be extensive. Our study is only preliminary, and was conducted to assess the physiological possibilities of differential sperm use by multiply mated females and to see if consistent patterns of sperm precedence occurs. In these lab studies, we were unable to test sexual selection theory.

CONCLUSIONS

We have shown that, under our sequential double mating regime, females of *P. glaucus* and *P. canadensis* generally produce larvae fertilized by the first rather than second male. Sperm mixing (with eggs fertilized by both species) and last male sperm precedence do occur at lower frequencies than first male sperm precedence. However, we had insufficient numbers of useful two-species matings of our lab and wild females to evaluate any potential conspecific cryptic sexual selection of the sperm types by females. Since it is so difficult to distinguish selection from sperm competition, it remains controversial (Simmons and Siva-Jothy 2001) whether females really have some influence or selection-capability in cases with conspecific sperm precedence (Howard 1999). However, the poorly understood role of sperm from different species in reproductive isolation in *Papilio* or other hybrid zones for polyandrous females may be significant (Arnold 1997; Endler 1998; Turelli *et al.* 2001; Howard *et al.* 2002; Via 2002; Andolfatto *et al.* 2003). It was evident in these studies that lab-reared males (even when fed an amino acid, salt, sugar elixir solution; as in Lederhouse *et al.* 1990) were still inferior to field-collected "wild"

TABLE 8. Egg viability for wild *Papilio glaucus*, wild *P. canadensis*, their reciprocal hybrids (females lab reared; males wild). Data represent the mean (± se) of all individual females (1995–2002).

	(# of females)	Total larvae	Total eggs	Egg Viability
P. glaucus	(246)	77.3 (5.1)	110.5 (5.7)	65.3 (1.6)%
P. canadensis	(305)	31.7 (2.3)	53.5 (2.9)	58.9 (1.7)%
Primary Hybrids				
Pg × Pc	(73)	108.5 (11.1)	167.1 (15.1)	62.3 (3.6)%
Pc × Pg	(17)	55.2 (13.0)	77.1 (16.6)	68.0 (6.2)%
Backcrosses (1997-2002);				
PgPc × Pc	(3)	118.0 (37.3)	148.3 (42.9)	78.5 (3.3) %
PcPg ×Pg	(6)	18.6 (9.9)	33.2 (10.8)	44.9 (12.2) %
PcPg × Pc	(20)	33.2 (7.0)	80.1 (10.9)	39.9 (5.6) %
Pg × PgPc	(1)	278	288	96.5 %
Pg × PcPg	(1)	17	143	12.0 %
F_2 Hybrids				
PcPg × PcPg	(2)	24.5	80.1	16.9 %
PgPc × PgPc	(1)	12	63	19.1 %

Papilio glaucus from populations in various states of the USA (FL, CA, OH, MD, VA, MO, MI)
P. canadensis from various populations in Canada and the USA (AK, WI, MI, NY, VT)
T-tests between *P. glaucus* vs. *P. canadensis* were all significant ($p < 0.01$) for total larvae, total. eggs, and egg viability. For hybrids (*Pg x Pc* vs. *Pc x Pg)* viabilities were not significantly different, but total eggs and total larvae were ($p < 0.01$). Mean egg viability of Backcross type *PcPg x Pc* (39.9%, n=20 families) was significantly lower than both parental types and both primary hybrids (all $p< 0.001$). Another two backcross types (n=6; and n=20 families) were not significantly different than parentals and primary hybrids for egg viability (genotypes with only 1 or 2 families were not compared).

males with respect to reproductive potential in hand-pairings for these *Papilio*. Differential larval food quality and natural puddling behavior of adult males (Pivnick and McNeil 1987; Boggs 1997a, 1997b) can affect male physiological virility and mating success and may largely account for the higher reproductive success of wild males than lab-reared males in our study.

Finally, the extent of hybrid vigor as seen in our F_1 hand-paired lab crosses (see also Donovan 2001; Scriber *et al.* 2003) may not occur in the natural field situation where hybrid males (Davies 1997) or natural enemies might differentially affect the fitness of hybrids (e.g. Mallet and Barton 1989) or backcrosses (Hagen and Scriber 1995; Dasmahaptra *et al.* 2002; Presgraves 2002). Recent examples of likely recombinant hybrid speciation in insects (Schwarz *et al.* 2005; Scriber and Ording 2005; see also Rieseberg *et al.* 2001) depend on introgressive hybridization. However, the role of sperm precedence in the recently apparent ineffectiveness of prezygotic barriers between *P. glaucus* and *P. canadensis* (Deering and Scriber 2002; Scriber 2002a; Donovan and Scriber 2003; Scriber *et al.* 2006) was not resolved by this study.

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